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XIAP is a promising new molecular target for the design of an entirely new class of cancer therapy to improve survival and quality of life of prostate cancer patients. New therapies targeting XIAP may prove to be especially effective to overcome apoptosis-resistance of prostate cancer cells. Using a powerful computational structure-based design strategy, we have designed and synthesized a new class of non-peptide small-molecule inhibitor of XIAP. The most potent compound binds to XIAP with a low nanomolar affinity and is potent in inhibition of cell growth in androgen-independent human prostate cancer cell lines. Furthermore, it is highly potent and effective in enhancing the activity of other anticancer drugs in human prostate cancer cells. Importantly, it has a low toxicity to normal cells. These compounds represent promising leads for further optimization toward our ultimate goal of developing a new class of anticancer drugs by targeting XIAP and promoting apoptosis in cancer cells.

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## Introduction

Androgen withdraw remains the only effective form of systematic therapy for men with advanced prostate cancer, with objective response in 80% of patients. Unfortunately, progression to androgen independence occurs within a few years in the majority of these cases. Despite extensive clinical trials, chemotherapy has limited antitumor activity, with an objective response rate of less than 50% and no demonstrated survival benefit. Thus, androgen-independent (hormone-refractory) disease is the main obstacle to improving the survival and quality of life in patients with advanced prostate cancer. There is an urgent need for novel therapeutic strategies for advanced prostate cancer by targeting the fundamental molecular basis of resistance of androgen-independent disease to chemotherapy.

Most of the current chemotherapeutic agents for advanced prostate cancer work by indirectly inducing programmed cell death or apoptosis in cancer cells. The aggressive cancer-cell phenotype is the result of a variety of genetic and epigenetic alterations leading to deregulation of intracellular signaling pathways. Such alterations include an impaired ability of the cancer cell to undergo apoptosis. Indeed, hormone-refractory prostate cancer is very resistant to apoptosis induced by chemotherapeutic agents and radiation. Thus, current and future efforts toward designing and developing new therapies to improve survival and quality of life of prostate cancer patients must include strategies that specifically target prostate cancer-cell resistance to apoptosis. Therefore, developing new and specific anticancer drugs that target critical apoptosis regulators by overcoming apoptosis of prostate cancer cells is a very exciting and fruitful area of research to improve the outcome of current anticancer therapies.

Inhibitor of apoptosis proteins (IAPs) have been identified as a class of central negative apoptosis regulators. XIAP is the most potent anti-apoptotic member among all the IAPs and has a key function in the negative regulation of apoptosis in both the cell surface death receptor- and the mitochondria-mediated pathways. Prostate cancer PC-3, DU-145 and LnCap cell lines have much higher levels of XIAP protein than normal prostate epithelial cells. XIAP has been implicated to play a key role in apoptosis-resistance of prostate cancer cells to chemotherapies. Because XIAP blocks apoptosis

at the effector phase, a point where multiple signaling pathways converge, new therapies targeting XIAP may prove to be especially effective to overcome apoptosis-resistance of prostate cancer cells and to develop an entirely new class of cancer therapy to improve survival and quality of life of prostate cancer patients.

Smac/DIABLO is a potent pro-apoptotic protein, which directly interacts with XIAP and other IAPs and promotes apoptosis by antagonizing the anti-apoptotic function of IAP proteins. Micro-injection of Smac protein was shown to promote apoptosis in prostate cancer cells. Three recent studies showed that short Smac-based peptides, consisting of the first 4 to 8 residues of the N-terminus of Smac tethered to a carrier peptide for intracellular delivery, sensitize various tumor cells *in vitro* for apoptosis induced by TRAIL or chemotherapeutic drugs and greatly enhance the anti-tumor activity of therapeutic agents *in vivo*. Importantly, Smac-based peptides show little or no toxicity to animals. These studies thus provide the important proof-of-concept that Smac-based small molecule inhibitors may have a great therapeutic potential for treating prostate cancer with XIAP overexpression.

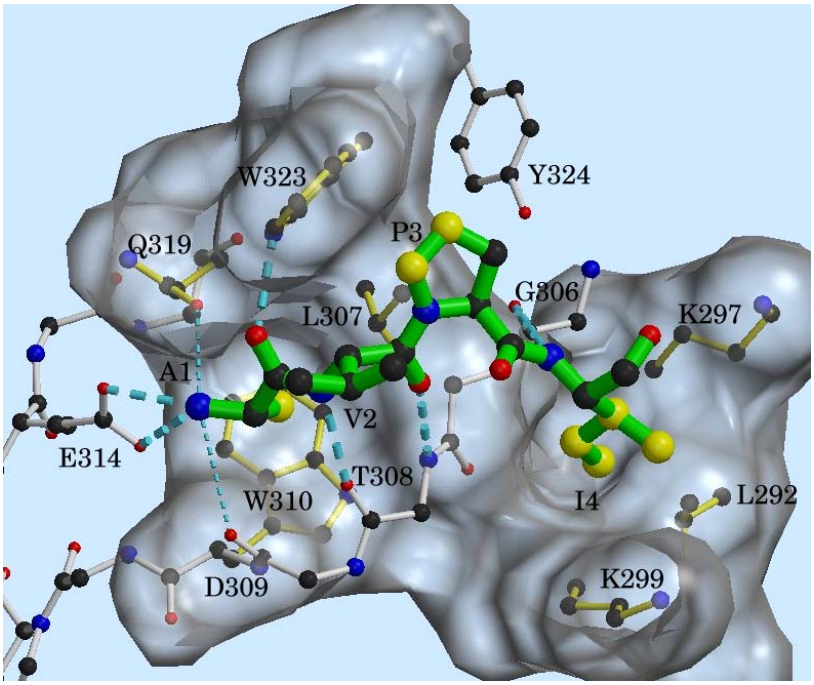
Peptide-based inhibitors have several intrinsic limitations to be developed as potential drugs, including poor cell-permeability and poor *in vivo* stability. For this reason, in this IDEA Development Grant, we propose to design and synthesize potent, non-peptide**,** cell-permeable, small molecule inhibitors of XIAP (Smac mimetics) and to test their therapeutic potential for the treatment of prostate cancer using a powerful structure-based design strategy based upon a class of most promising non-peptide small molecule inhibitors we have already discovered in our laboratory. Successful carried out, our studies will represent an exciting step and lay the foundation for developing an entirely new class of anticancer drugs by targeting a central apoptosis regulator protein. It is predicted that such a drug will have very few side effects and will be able to significantly improve the outcome of current clinical treatment protocols by specifically overcoming apoptosis-resistance of prostate cancer cells to chemotherapeutic agents through targeting the fundamental molecular basis of apoptosis-resistance in prostate caner cells.

**Body of the report:**

*Design of non-peptide, monovalent and bivalent Smac mimetics*

Since our ultimate goal is to develop a potent Smac mimetic for the treatment of androgen-independent human prostate cancer, we have now expanded our initial efforts, which primarily focused on one class of compounds, to additional classes of compounds.

**Figure 1.** X-ray structure of Smac in complex with XIAP BIR3. Carbon atoms are shown in green for Smac AVPI peptide. Hydrophobic carbon atoms on the side chain of Alanine, Proline ring and Isoleucine in Smac peptide are shown in yellow. Hydrogen bonds are depicted in light-blue dashed lines. Oxygen and nitrogen atoms are shown in red and blue, respectively. Carbon atoms in XIAP BIR3 protein are shown in black.



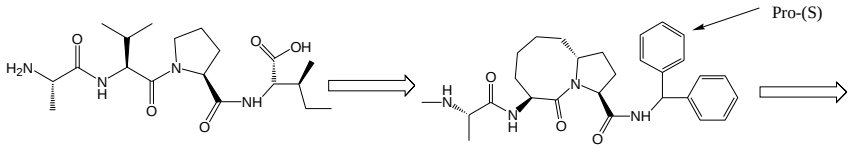

High-resolution, experimental three-dimensional (3D) structures of the BIR3 domain of XIAP in complex with Smac protein and peptide have been determined (**Figure 1**).The N-terminal tetrapeptide of Smac (Ala-Val-Pro-Ile) recognizes a surface groove on the BIR3 domain of XIAP through several hydrogen-bonding interactions and van der Waals contacts. The interaction between BIR3 and caspase-9 has also been shown to

involve four residues (Ala-Thr-Pro-Phe, or AVPI) on the amino terminus of the small subunit of caspase-9 to the same surface groove on the BIR3 domain. Several recent studies have convincingly demonstrated that Smac promotes the catalytic activity of caspase-9 by competing with caspase-9 for the same binding groove on the surface of the BIR3 domain.

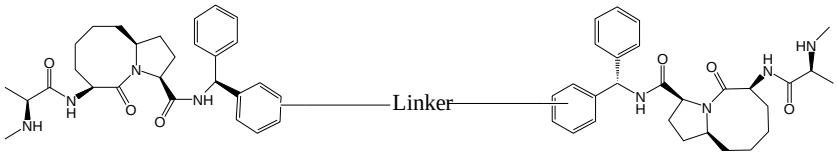
Unlike most protein-protein interactions, the Smac-XIAP interaction is mediated by only four amino acid residues (AVPI) on the Smac protein and a well-defined surface groove on the BIR3 domain of XIAP. The $K_d$ value of Smac peptide AVPI to XIAP ($K_d$ =400 nM) is essentially the same as the mature Smac protein ($K_d$ = 420 nM). This well-defined interaction site is ideal for the design of non-peptide, drug-like small molecules that mimic the binding of Smac to XIAP. Indeed, our NMR and computational modeling studies have shown that Embelin binds to this site.

Based upon the X-ray structure of XIAP BIR3 in complex with Smac, we have designed **SM-122** as a conformationally constrained Smac mimetic to closely mimic the binding of Smac to XIAP BIR3 domain (**Figure 2**). Our cyclization strategy converts the two natural amino acids (valine and proline) into a non-amino-acid, bicyclic, lactam ring system, and the resulting Smac mimetics become non-peptide, i.e. there is no amino-acid bond in these Smac mimetics (**Figure 2**). Using our established fluorescence-polarization-based (FP-based) competitive binding assays, we have determined that **SM-122** binds to recombinant XIAP BIR3 protein with a $K_i$ value of 27 nM, and to recombinant XIAP BIR2 protein with a $K_i$ value of 2 μM. These data suggest that **SM-122** binds to XIAP BIR3 preferably but also has a significant binding to BIR2 domain of XIAP.

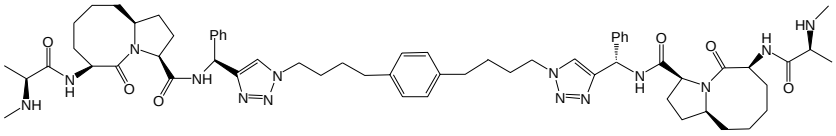
**Figure 2**. Designing conformationally constrained, non-peptide, bivalent Smac mimetics.




**Smac AVPI peptide** **SM-122** ( A potent, non-peptide Smac Mimetic)



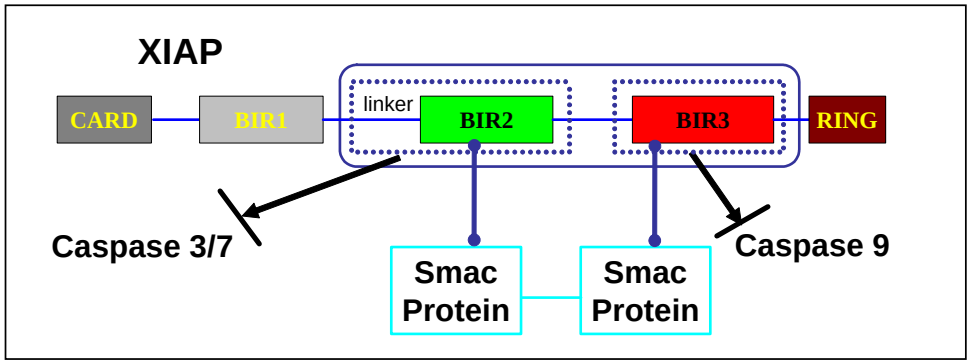

Basic template for bivalent Smac mimetics



**SM-164** (A Conformationally constrained non-peptide bivalent Smac Mimetic)

XIAP contains three baculoviral IAP repeat (BIR) domains (**Figure 3**). Its BIR2 domain, together with the linker before the BIR2 domain, binds to effector caspases-3 and -7 and inhibits their activity and its BIR3 domain binds to and inhibits an initiator caspase-9. In this manner, XIAP efficiently inhibits apoptosis by binding to and inhibiting the activity of not only caspase-9 but also caspase-3 and -7, whose activity is crucial for execution of apoptosis. We hypothesize that small-molecule inhibitors designed to concurrently target both the BIR2 and BIR3 domains of XIAP will be much more efficient in antagonizing XIAP in cells. We predict that such compounds, which are called "**bivalent Smac mimetics**" will achieve a higher binding affinity to XIAP and will be far more effective than agents that target only the BIR2 or the BIR3 domain in promoting apoptosis in cancer cells.

**Figure 3**. XIAP inhibits caspase-9 through its BIR3 domain and caspase-3/-7 through its BIR2 domain together with the linker before BIR2. Smac protein forms a dimer and binds to both BIR2 and BIR3 domains in XIAP and effectively antagonizes the function of XIAP.




Using **SM-122** as the monovalent Smac mimetic, we have designed bivalent Smac mimetics to target both the BIR2 and BIR3 domains of XIAP. Analysis of our predicted binding models of **SM-122** to XIAP BIR3 showed that the phenyl ring at the pro-(*S*) position in **SM-122** can be used for chemical tethering (**Figure 2**), since this phenyl ring is exposed to solvent and is not in contact with any protein atoms. A series of bivalent Smac mimetics were designed with the basic template structure shown in **Figure 2**. To date, we have synthesized more than 10 such bivalent Smac mimetics with different types of chemical linkers. **SM-164** is one of the most potent and promising inhibitors (**Figure 2**).

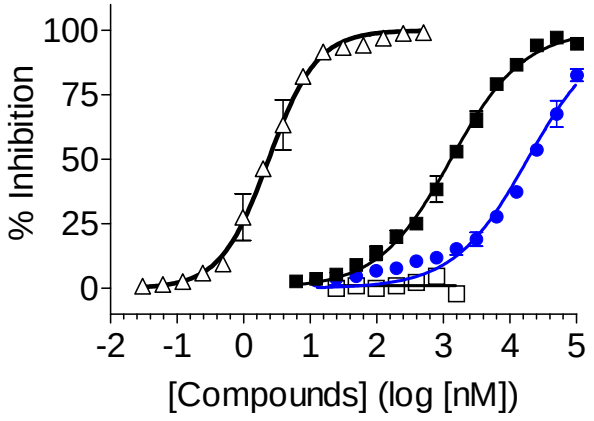
**SM-164** was determined to bind to XIAP protein containing both the BIR2 and BIR3 domains (residues 120-356) with an $IC_{50}$ value of 1.9 nM (estimated Ki value equal to 200 pM) in our competitive FP-based assay (**Figure 4**). Of note, the hill-slope of the binding curve is 1, suggesting the formation of 1:1 complex between **SM-164** and XIAP. In addition, gel-filtration experiments showed that our designed bivalent Smac

mimetics form 1:1 complex with the XIAP protein containing BIR2 and BIR3 domain (data not shown), consistent with our FP-based binding results.

**Figure 4**. Competitive binding of designed monovalent and bivalent Smac mimetics to recombinant XIAP L-BIR2-BIR3 protein as determined using our FP-based binding assay.

**Compounds** [$IC_{50}$ ± SD [nM] ($K_i$ ± SD [nM])]

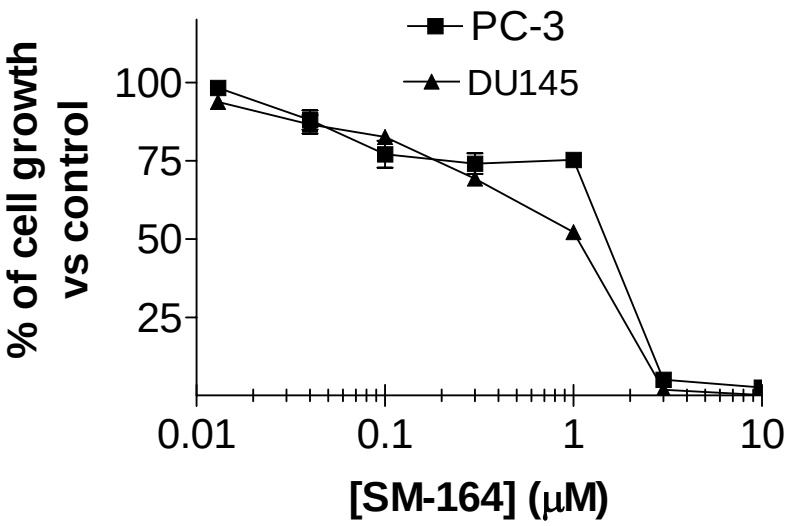
- ● **AVPI** [18,063 ± 1,800 (2,064 ± 205)]
- ■ **SM-122** [1,396 ± 122 (159 ± 14)]
- △ **SM-164** [1.9 ± 0.5 (~0.2 ± 0.06)]
- □ **SM-149** [> 20,000]




In comparison, **SM-122** has an $IC_{50}$ value of 1.4 μM (Ki = 159 nM) to XIAP protein containing BIR2 and BIR3 domains and the AVPI Smac peptide has an IC50 value of 18 μM ($K_i$ value of 2.1 μM). Hence, **SM-164** is 800-times more potent than the Smac AVPI peptide and 28-times more potent than the monovalent **SM-122**. **SM-164** represents the most potent Smac mimetic discovered to date. Since **SM-164** is non-peptide, it is predicted that it will have major advantages over peptide-based Smac mimetics for its cell-permeability, *in vivo* stability and bioavailability.

*Bivalent Smac mimetics potently inhibit cell growth in PC-3 and DU-145 cells*

**Figure 5**. Inhibition of cell growth by **SM-164** in PC-3 and DU-145 cell lines, as determined using WST-based assays when cells were treated for 4 days.



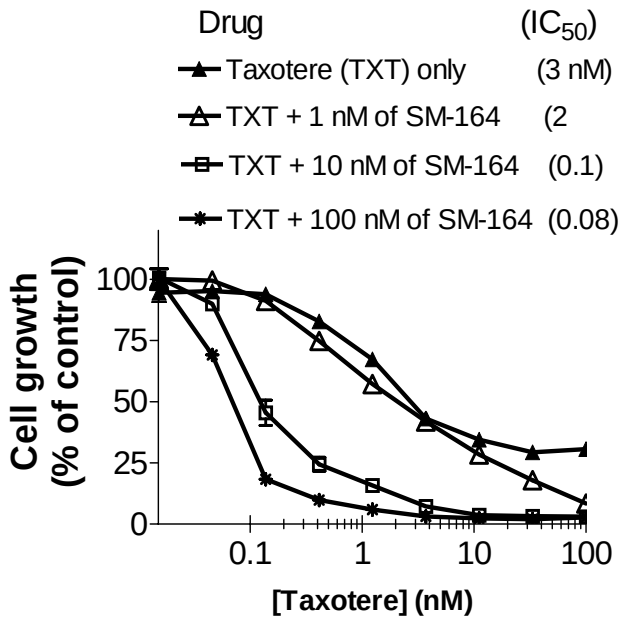

**SM-164** was evaluated for its ability to inhibit cell growth in PC-3 and DU-145 cells. **SM-164** quite potently inhibited cell growth in both PC-3 and DU-145 cells with $IC_{50}$ values of 1.5 and 1 μM, respectively (**Figure 5**). In direct comparison, **SM-164** is at least 50-times more potent than the monomeric **SM-122** (data not shown). This is consistent with its higher binding affinity to XIAP for **SM-164** than for **SM-122**.

***SM-164*** *is highly effective in enhancing the activity of taxotere in human prostate cancer PC-3 cells*

Since bivalent Smac mimetics such as **SH-164** are expected to effectively antagonize the inhibition of XIAP to not only caspase-9 but also caspase-3/-7, we predict that they may be able to greatly enhance the activity of other chemotherapeutic agents. To this end, we have evaluated **SM-164** for its ability to enhance the activity of taxotere, which is an agent approved by the FDA as a treatment in combination with

prednisonefor patients with androgen independent (hormone refractory) metastatic prostate cancer.

**Figure 6**. **SM-164** strongly synergizes with taxotere in the PC-3 human prostate cancer cell line. Cells were treated for 4 days and cell growth was determined using the WST-based assays when



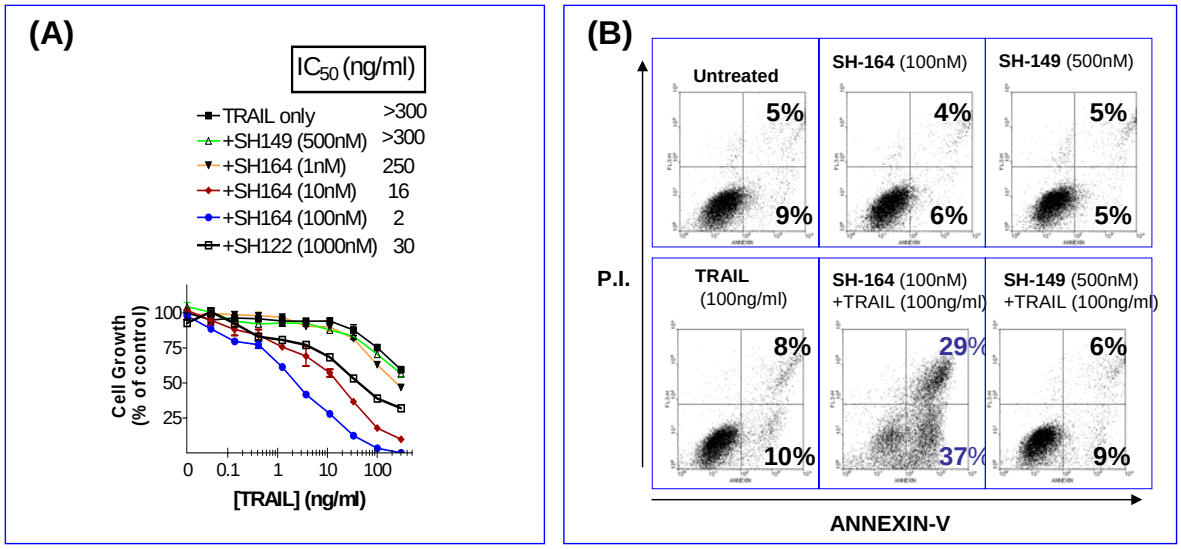

Our results show that **SM-164** is highly effective in enhancing the activity of taxotere in androgen-independent, PC-3 prostate cancer cells (**Figure 6**). Taxotere alone has an $IC_{50}$ value of 3 nM in inhibition of cell growth. Combination with as low as 10 nM of SM-164 enhance the activity of taxotere by 30-times. Of note, since 10 nM of **SM-164** has no effect on its own, **SM-164** in combination with taxotere achieves a truly synergistic effect. Based upon the exciting in vitro results, we plan to carry extensive in vivo studies to further evaluate the therapeutic potential of **SM-164** in combination with taxotere as a potentially new therapeutic strategy for the treatment of androgen, independent human prostate cancer.

***SM-164*** *is highly effective in enhancing the activity of TRAIL in human prostate cancer PC-3 cells*

Another agent we have evaluated in combination with **SM-164** is TRAIL (TNF-related apoptosis-inducing ligand). TRAIL was shown to be effective in inducing apoptosis in some cancer cells and importantly has a low toxicity to normal cells and tissues. TRAIL has been considered as a promising new anticancer therapy for the treatment of human cancer and is currently in clinical development. Unfortunately, TRAIL resistance is common in both preclinical and clinical studies.

PC-3 prostate cancer cells are insensitive to TRAIL, having $IC_{50}$ values >300 ng/ml in the cell growth inhibition assay (**Figure 7A**). **SM-164** greatly enhances the activity of TRAIL in the PC-3 prostate cancer cell line. Combination of 10 nM and 100 nM of **SM-164** with TRAIL decreases the $IC_{50}$ value of TRAIL from >300 ng/ml to 16 and 1 ng/ml, respectively. Importantly, since **SM-164** alone has minimal activity at as high as 500 nM, the dramatic enhancement in the activity of TRAIL by **SM-164** in the PC-3 cell line is also a true synergistic effect. **SM-149**, the inactive control, has no enhancement on the activity of TRAIL, indicating that the effects by **SM-164** are specific and correlate with its potent binding to XIAP protein.

**Figure 7**. Bivalent Smac mimetic **SH-164** dramatically synergizes the activity of TRAIL in human prostate cancer PC-3 cell line. (**A**). PC-3 cells were treated for 4 days and cell growth inhibition was determined by the WST-based assay; (**B**). PC-3 cells were treated for 12 hours and apoptosis was determined by flow cytometric analysis by Propidium iodide (P.I.) and ANNEXIN-V double staining.

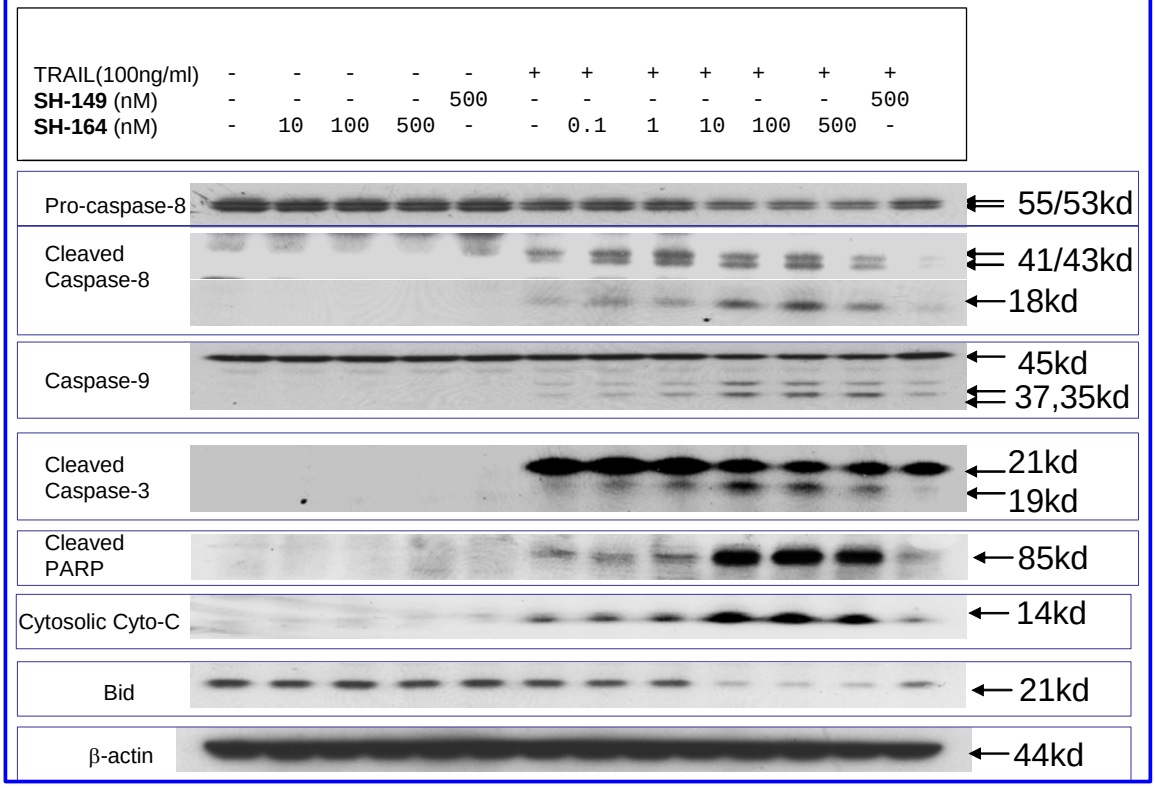



***SM-164*** *enhances the apoptosis induced by TRAIL in the PC-3 cells*

We have further evaluated **SM-164** for its ability to enhance apoptosis induction by TRAIL in PC-3 cells using Annexin-V/PI double staining. It was found that while treatment of PC-3 cells by TRAIL alone at 100 ng/ml for 24 hours resulted in 18% apoptotic cells, the combination of 100 nM of **SM-164** with 100 ng/ml TRAIL increased the apoptotic cells to 65% (**Figure 7B**). There was no increase in apoptotic cells when TRAIL was combined with 100 nM of the inactive control **SM-149** as compared to TRAIL alone.

Western blotting analysis showed that there is a marked increase in the processing of caspase-8, caspase-9 and caspase-3, and cleavage of PARP (**Figure 8**). Our Western analysis provided strong evidence that SM-164 greatly enhances the activity of TRAIL at the molecular level.

**Figure 8**. Western blot analysis of caspases, cleaved PARP, Cyto-C release and Bid cleavage in PC-3 cells treated by TRAIL, or **SM-164**, inactive control SM-149, or combination for 10 hours.




***SM-164*** *is not toxic to normal prostate epithelial cells*

**SM-164** was tested for its toxicity to normal epithelial and fibroblast cells. **SM-164** is much less toxic to normal cells and displays an $IC_{50}$ value of >10 μM in both normal prostate epithelial cells and normal fibroblast cells.

**Research Accomplishments:**

(1). We have designed and synthesized a novel class of non-peptide small-molecule inhibitors of XIAP. The most potent lead compound, **SM-164**, has an $IC_{50}$ value of 1.9 nM, 8000-times more potent than the natural Smac peptide. **SM-164** is probably the most potent Smac mimetic discovered to date.

(2). **SM-164** potently inhibits cell growth in PC-3 and DU-145 androgen-independent human prostate cancer cell lines**.**

(3). **SM-164** is highly potent and effective to sensitize PC-3 prostate cancer cells to taxotere and TRAIL at low nanaomolar concentrations and achieves a true synergistic effect.

(4). **SM-164** a minimal toxicity to normal prostate epithelial cells.

(5). Extensive in vivo studies are ongoing.

(6). Detailed mechanism studies are ongoing.

**Reportable Outcomes:**

(1). Two manuscripts described the design, synthesis and biochemical and biological characterization of this class of compounds has been finished. One has been submitted and the second one will be submitted shortly.

(2). An invention disclosure has been filed with the Technology Transfer Office at the University of Michigan. A patent application will be filed soon.

**Conclusions**: Smac mimetics such as **SM-164** represent highly potent small-molecule antagonists to target XIAP and is the most potent small-molecule inhibitor of XIAP discovered to date. **SM-164** is extremely effective to sensitize androgen-independent human prostate cancer cells to other therapeutic agents such as taxotere.

In addition, the most potent new analogue is effective in inhibition of cell growth in human prostate cancer cells with high levels of XIAP. Further optimization for this class of compounds and extensive testing may ultimately lead to an entirely new class of anticancer therapy for the treatment of androgen-independent human prostate cancer by targeting XIAP.